Spring 3-12-2018

Factors Affecting the Parasitism of Gregarine Species in Grasshoppers In Western Nebraska

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Abstract

We conducted a survey of the species of parasites found in grasshoppers around the area of Dunwoody's Pond to investigate whether there were any species specific parasitic relationships occurring there. We collected grasshoppers from several areas around Dunwoody's Pond totaling 15 species including *Melanoplus bivittatus* (Say 1825) , *Melanoplus sanguinipes* (Fabricius, 1798) , *Melanoplus differentialis* (Thomas 1865), and *Melanoplus femurrubrum* (De Geer 1773), along with others (Seymour et al., 2009) and compared the parasites found in them. This study is similar to one conducted in South America by Lloyd in 1951, and we hoped to find connections between species to support or disprove cospeciation hypotheses (Page, 1993) and to determine if factors such as the developmental stage or the sex of the host insect affected the prevalence of parasites in the hosts. We had expected to find mites on the external shell of the grasshoppers and horsehair worms in the body cavities of the grasshoppers based off of our preliminary research and our literature search (Rees, 1973), (Hanelt et al., 1999). Our results indicate a distinct lack of species specific parasitism as well as a complete absence of any mites or horsehair worms. We found multiple species of gregarines including *Amoebogregarina nigra* Clopton, 1999, two unidentified species of gregarine and maggots, across several host species of grasshoppers. Analysis of this data supports a hypothesis of non-species-specific parasitism and indicates that two host factors (development stage and sex) are statistically insignificant to parasite prevalence in grasshoppers.

Introduction

Parasitism is a specific subset of commensalism- the act of one organism gaining a benefit from another to the detriment of the host. Put another way, one organism uses another (the 'host') as its ecosystem and its source of nutrition (Zelmer, 1998). These organisms can take many forms,

from the greatest of mammals to the tiniest of bacteria and quite literally everything in between. While these relationships are critically important to all ecosystems on Earth, they are often over looked and understudied. Public and scientific interest in and deep concern for biodiversity has been on a steady increase for many years, and broad-spectrum surveys to identify species in threatened areas have been common since the 1990s, if not earlier (Hawksworth, 2002), (Fenchel and Finway, 2004). Despite this, wide ranging surveys of parasites are typically conducted only on specific species (either of hosts or of parasites) of interest, rather than as direct aides to and records of biodiversity. This host-directed approach comes as no surprise when viewed through the lens of parasitism's earliest assumptions regarding host specificity.

Parasitologists have long observed the high host specialization of parasites and hypothesized that they would be ill fitted for survival outside of their traditional host (Thompson, 1994). This hypothesis created the parasite paradox- that such an evolutionary "dead-end" should create an evolutionary environment that is non-conducive to host switching. This theory contradicts scientific observations, such as those of the host switching of parasites in genera *Haemoproteus* and *Plasmodium* between several species of birds in Europe (Bensch et al., 2000). This coevolutionary "arms race" has been experimentally demonstrated in the genetic interactions between the crustacean *Delphina magna* and the bacterium which parasitizes it, *Pasteuria ramose*. In that study, the parasites adapted to a certain genetic line of host were shuffled with other hosts to demonstrate their adaptivity outside the most specific genetic relationship formed. Additionally, two strains of the parasites were shuffled into two cloned lines of the host, demonstrating how each parasite line was able to adapt to the specific idiosyncrasies of the hosts they were placed with. In both cases, the parasite was equally infestive in the new host as in the old. This adaptation demonstrates the parasite paradox perfectly- despite being removed from their idealized host, the

parasites were able to adapt and thrive equally well in the new host, which according to the historical understanding of parasitology should not be the case (Little et al., 2006). Agosta et al. discuss this in a 2010 paper, noting that the ability of an organism to utilize resources and the possession of phenotypic variation (termed "ecological fitting")- the same traits which make parasites such successful specialists- can in times of duress be redirected to allow for new host fitting when further specialization towards a current host is unsustainable.

Recent research such as that done by S. Johny et al. (2000) suggest that host switching and adaptation of a parasite to new hosts are not only possible, they are likely in situations where a parasite and a host are brought together in new combinations, such as through the action of climate change or habitat loss. The Stockholm paradigm explains such changes as ecological fitting in action- the changing of hosts as resource situations demand it, (Brooks, 2014) which under previous models was regarded as the exception rather than the rule (Hoberg, 2015). This phenomenon of adaptation has wide ranging impacts on human society and health, as many of the host switches thus described could potentially be from animals into humans, leading to new epidemics of disease or parasite infestation in people (Brooks, 2014). Not all host switching events are detrimental to humans, however. As global concern about pesticides and chemicals in food are rising, biocontrol of agricultural pests that will not be transmitted to humans are in high demand (Pushkala, 2000).

There are 108 grasshopper species commonly found in Nebraska today (Brust, 2008). Of these, some only occur in specific localities, while others span the length of the state. There are almost 30 that can be found in the western reaches of the state, but the five most common species in the area near Dunwoody's Pond (a private irrigation pond on a farm outside of Ogallala, NE) are *Melanoplus lakinus* (Scudder, 1879) *, M. femurrubrum, M. bivittatus , M. sanguinipies* and *M.*

differentialis. These can be differentiated from one another by morphological features such as size, color, markings and wing features (Brust, 2008). *M. differentialis* is one of the most commonly found, and has been an experimental host for gregarines for many years (Allegre, 1948).

Gregarines are parasitic organisms of the order Eugregarinorida, which contains 244 genera with 1656 described species (Clopton, 2002). Gregarina are described mainly on the basis of morphology, with the descriptions and terms for their plane shapes outlined by Clopton in 2004. There are three main suborders: blastogregarinorina, aseptatorina, and septatorina. They are differentiated from one another on the basis of size, life cycle features (such as whether they reproduce asexually or sexually, or how many sporocytes are produced from an oocyst), and morphological features such as the presence or absence of a septum or the presence and shape of an epimerite. Blastogregarinorina do not engage in syzygy or produce gametocysts, releasing 10-16 naked sporocysts instead. Aseptatorina do not have a septum (and therefore do not have a deuteromerite or a protomerite), and may or may not have an epimerite or mucron depending on the species. Septatorina have a septum dividing the deuteromerite and the protomerite, and generally have an epimerite as well (Clopton, 2002). Using this information as a background, we took a survey of the parasite prevalence and diversity around Dunwoody Pond and analyzed statistics relevant to the species we studied and their hosts.

Methods and Materials

We collected grasshoppers from Dunwoody Pond near Ogallala, Nebraska. Grasshoppers were collected using nets in locations near the pond, including along the edges of the pond, in the grass field bordering it, and in the grasses bordering the wheat field along the pond. Grasshoppers were then returned to the lab where species, maturity (nymph or adult), sex, length and weight

were recorded. Once collected the grasshoppers were kept at a low temperature to preserve them until they could be dissected. During the latter half of the project the grasshoppers were first sequestered into individual jars before being stored to prevent cross contamination. They were fed grasses to encourage defecation, which was saved for potential sporocyst collection.

The grasshoppers were then euthanized in ethanol and examined for external parasites, which were counted and recorded. The grasshoppers were dissected under a dissecting microscope to examine for internal parasites. The gastrointestinal tracts of the grasshoppers were removed and submerged in an insect saline mixture composed of one part NaCl to one hundred parts distilled water. The body cavity of the insect was inspected under the dissecting microscope to look for parasites outside of the gastrointestinal tract.

The gastrointestinal tract was then examined under the dissecting microscope for parasites. The inspection began at the crop, and consisted of observing the intact organ, and then pulling it apart to observe internal structures and the contents of the organ. In cases where a higher resolution was needed for precise identification, a light microscope was used. Specimens with parasites were photographed and stored in liquid nitrogen with the host's body and the gastrointestinal tract preserved separately for later study. Samples of the gregarines found were either preserved in situ with the digestive tracts or mounted on slides for further staining and observation. Fecal samples of highly infected host specimens were preserved in collection jars for later sporocyst collection.

Sporocyst collection was undertaken according to the methods laid out by Clopton et al. on the website hotelintestine.com. Fecal samples from infected hosts were submerged in insect saline and allowed to soften for 2 hours, during which time they were placed on a laboratory shaker. After this period, they were further broken apart by hand using paintbrushes and

observed under a dissecting microscope, with light microscopes used for confirmation of ambiguous specimens. Had specimens been discovered, they would have been placed on sterilized 6mm dark paper disks and placed in a 60-mm center-well organ cultuer dish, and the outer well of the dish filled with a hydrating gel to prevent dehydration. These would have then been covered to protect the humidity level and allowed to incubate at ambient temperatures. Any observed oocysts would have been observed and recorded.

Results

We collected a total of 143 grasshoppers from 15 species. From those hosts, we found a total of 42 infected individuals, for an overall parasite prevalence of 0.29371. We found three morphologically distinct species of gregarines in these hosts, and in three of the insects we found maggots. All three of the gregarine species were found inside the midgut of the insect, with very highly infested host specimens having parasites in the gastric ceca in addition to those in the midgut.

The first gregarine is very distinctive, a generally pyriform shape with a deuteromerite that is shaped like a rounded rectangle, an elongated protomerite and a distinct epimerite (fig. 1). These were found in *Melanoplus femurrubrum*, *M. bivittatus*, and *M. differentialis* specimens. It was identified as *Amoebogregarina nigra*.

The second gregarine found is linearly panduriform, with a narrow and elongated deuteromerite, with a slightly wider protomerite (fig. 2). These gregarines were found in *M. sanguinipes* and *M. femurrubrum* specimens. It was not identifiable using morphology in the field and attempts to cultivate sporocysts in lab for further identification were unsuccessful. It remains unidentified at the species level and may be a new species.

The final gregarine found is obpanduriform in shape, with a large, rounded deuteromerite, a small bulbous protomerite, and a thin epimerite visible (fig. 3). It was found across almost all species, including *M. fermurrubrum, M. differentialis, M. sanguinipies, M. lakinus,* and *M. bivittatus*. Attempts to collect sporocysts in the lab were also unsuccessful, and it remains unidentified. It may also be a new species.

Additionally, maggots (fig. 4) were found in three of the hosts. These hosts were from the species *M. differentialis*, *M. femurrubrum*, and *M. bivittatus*. They were found in the thoracic cavity of the insects, and in each case presence of the maggot was accompanied by the presence of gregarines in the midgut and occasionally the gastric ceca. The maggots were identified as larvae of flies in the genus Lespesia using morphology and a host based key (Arnaud , 1978).

Fig. 1. A. nigra, found in M. differentialis

Fig. 2. Gregarin sp.1, found in M. sanguinipies

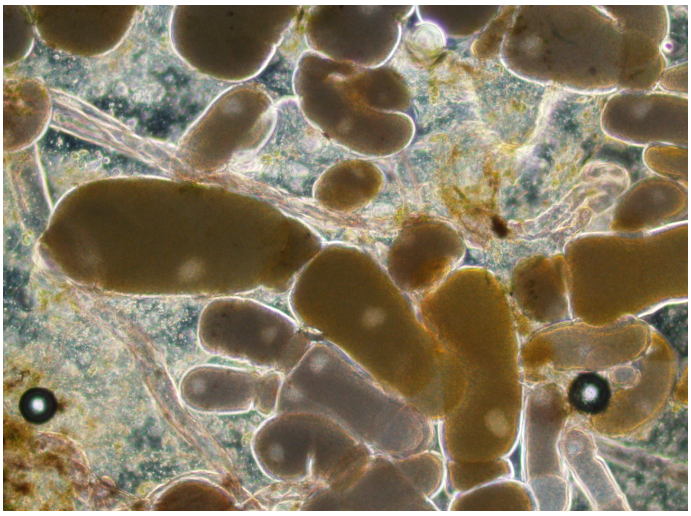

Fig. 3. Gregarine sp. 3, found in M. femurrubrum

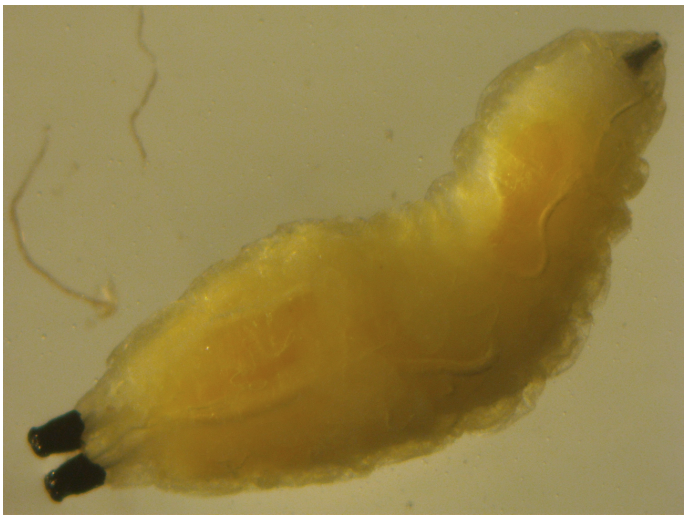

Fig. 4. Maggot of the genus Lespesia, found in M. differentialis

To determine the statistical validity of our data, we did a comparison proportions test of the data we collected to decide if factors such as the insect's sex or its developmental stage (ie adult or nymph) impacted its parasite prevalence. The analysis of developmental stage data is recorded in charts 1a and 1b, while the analysis of sex data is recorded in charts 2a and 2b. The average level of infestation between developmental stages and between sexes are demonstrated in the graphs below.

Maturation

Maturation	Infected	Pop.	Prevalence
adult	25	76	0.3289473684
nymph	16	46	0.34782087

Chart 1a. The number of grasshoppers divided by maturation level and the prevalence of parasites in those categories.

Analysis

Difference	1.88735016 %
95% CI	-15.9433 to 20.4983
Chi^2	0.045
DF	1
Significance level	P = 0.8313

Chart 1b. Analysis of the data with chi^2 value and significance level.

Sex

Sex	Pop.	Infected	Prevalence
F	48	17	0.3541666667
M	74	24	0.3243243243

Chart 2a. The number of grasshoppers divided by sex and the prevalence of parasites in those categories.

Analysis

Difference	2.98423424 %
95% CI	-14.8186 to 21.3568
Chi^2	0.115
DF	1
Significance level	P = 0.7342

Chart 2b. Analysis of the data with chi^2 value and significance level.

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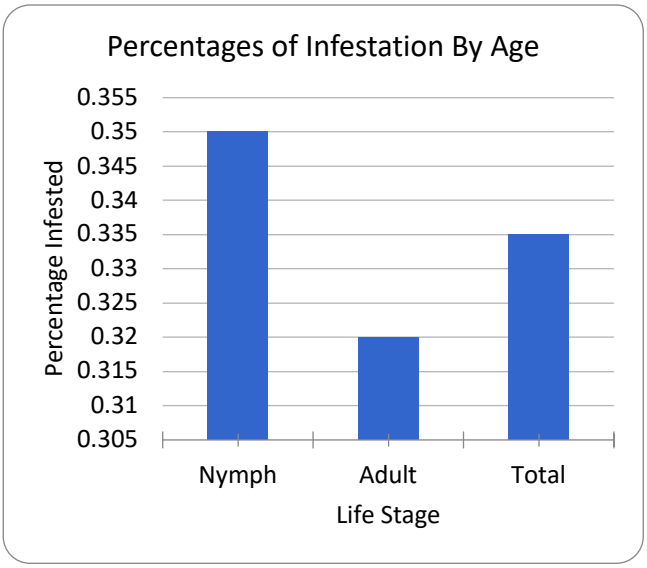

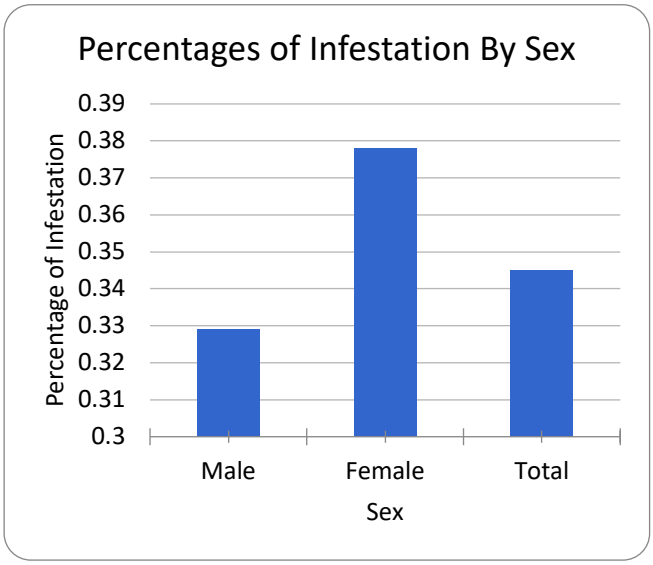

Conclusion

This survey was intended to inform about the variety of parasites to be found in grasshoppers in the area surrounding Dunwoody Pond, and the factors which may affect their prevalence in the host population. From our collection, we were able to identify three distinct species of gregarine and one species of maggot. One gregarine was identified as *A. negra,* while the maggots are identified as being in the genus Lespesia (Arnaud , 1978). The other two species

of gregarine remain unidentified. Though we collected fecal samples from specimens that were infested with the unidentified gregarines, no sporocysts were recovered, which prevented further identification efforts. This could be due to the method in which they were collected and stored or due to the amount of time which passed before the analysis could be conducted. Without lifecycle information, further identification of these two unknown gregarines will be dependent on future collection efforts and genetic analysis.

Our analysis of factors which might influence the prevalence of parasite infestation also yielded mixed results. We collected data on the grasshoppers' sizes, level of maturity, sex and species, but due to the random nature of collection we decided to do our analysis on the four most heavily collected species- *Melanoplus bivittatus*, *Melanoplus sanguinipes*, *Melanoplus differentialis*, and *Melanoplus femurrubrum*. We wanted to focus on factors which would be conserved across all species collected, and since the different species we collected have a wide range of size (weights ranged from <0.1g to greater than 1.2g, and ranging in length from 1.03cm to 3.82cm), we decided to use differences in sex and in maturity as our variables.

After analysis, we found that the data on prevalence relative to either sex or maturity indicated that neither variable is statically relevant across the five species we tested. This finding is in line with the study we referenced which found that sex in arthropod hosts was not a statistically significant variable in parasite prevalence (Letitia et al., 2000). One likely explanation for this is that in insects there is little behavioral difference between the sexes, partially due to lack of male/female specific hormones. In mammals, risk taking behavior is influenced by chemical differences in the sexes including the levels of hormones such as testosterone, which leads to many behavioral differences. Since there is not a strong behavioral difference between the sexes in

insects, the rates of infection are not affected by factors such as risk taking behavior, so there is not a statistical difference between sexes.

The analysis of the differences between adults and nymphs indicates that they were also not statistically different. This can be explained in a few different ways. Nymphs, while smaller than adults, still eat the same foods and thus have the same general exposure to parasite eggs in the environment as adults do. Since this study was only looking for the presence and diversity of the parasites, rather than the levels of infestation in individual hosts, the level of exposure is among the most important factors determining the data that we collected. This assumes that survivability of the gregarine in both adult and nymph is comparable- further study including the degree of infestation in individuals divided into the different age groups (1st through 6th instars of nymphs along with mature adults) would shed light on the issue of varying parasite densities as related to the age of the insect.

Additionally, since this study was conducted from mid to late August, most of the nymphs that we collected are later instars, mainly 5th and 6th. At this later stage of development, they would have had time to become exposed to the parasites and are also developmentally more similar to adults than the earlier instars, when they are smaller in size and have not fully developed internally. This fact most likely contributed to the very similar statistical findings between adults and nymphs. At earlier periods in their growth, it is likely that the parasite load per nymph would be lighter.

There were a few areas where error was possible in this study. To begin with, the nature of learning creates a learning curve, and it is possible that in our early results we missed seeing parasites due to lack of experience in dissecting arthropods and identifying parasites. Additionally, identification of grasshoppers is complex and often based on morphological traits that can vary between individuals or be easily mistaken for one another, which could skew our results if they

were to be broken down by species. These sources of error were minimized as much as possible, but should be acknowledged as potentially affecting our results.

The information we collected only brushes the surface of what can be found at Dunwoody Pond. It could be augmented by returning to the same location next year and collecting more larger and more equally distributed sample sizes of insect hosts in order to facilitate better statistical analysis; we can also attempt to collect more equal numbers of nymphs from all stages and adults to create a separate analysis of nymph parasite burden from adult parasite burden. Additionally, if we were to return, gathering intensity data in addition to prevalence would be invaluable information for analysis. The parasites found in other Orders of hosts can also be studied in these and similar ways to gain an understanding of how the parasites interact between not just similar species and genera but also more genetically distant relatives. Finally, conducting wider ranged studies (ie, at locations in the countryside around Dunwoody's Pond) can give insight into the distances that hosts and parasites can travel and how that travel affects them and their relationships to one another. Although this survey was successful in elaborating on what can be found around Dunwoody, continuing the research into the future can provide us with valuable data on gregarines and their relationships with grasshoppers and their environments.

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